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Air Force Research Laboratory Integrated Omics Research

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ABSTRACT Integrated Omics research capabilities within the Air Force Research Laboratory began in 2003 with the initiation of a Defense Technology Objective project aimed to identify biomarkers of toxicity occurring within the warfighter as a preclinical indicator. Current methods for determining toxic exposures are not responsive enough or created available for deployment to prevent serious health effects. Using Integrated Omics (Genomics/Epigenetics, Proteomics, and Metabonomics) for biomarker discovery, we have identified specific molecular markers which, once validated, could be used for real-time or near-real-time monitoring of the human response to uncharacterized exposures. The determination and use of validated biomarker sets, when installed on a fieldable biomonitor system, could allow fast determination of subclinical organ damage in response to chemical exposures. Since initiation of this program, our group has applied Omics technologies for biomarker discovery in a number of toxicology and human performance projects, including jet fuel exposures and cognitive fatigue.

INTRODUCTION

One of the goals of Air Force Research Laboratory (AFRL) is the development of new methods to assess warfighter performance by using advanced biotechnologies that could provide near-real-time detection of early state changes in health and performance status. As a part of this thrust, Integrated Omics research capabilities (genomics, proteomics, and metabonomics) within the Air Force began in 2003 with the initiation of a Defense Technology Objective (DTO) project. The research project (MD.34 Biotechnology for Near-Real-Time Predictive Toxicology) aimed to identify biomarkers of toxicity occurring within the warfighter as a preclinical indicator. The DTO was primarily generated in response to unexplained physical symptoms experienced by deployed military personnel during Operation Desert Storm in 1990. As a result of concern regarding these symptoms, the National Science and Technology Council Presidential Directive 5 was issued in 1998, and focused on deployment health preparedness. This was followed by the Defense Intelligence Report DI-1816-8-99 (Medical Intelligence Assessment of Deployment Environmental Health Risks) in 1999.

The Defense Intelligence report stated "Personnel deployed in support of missions ranging from war to operations other than war may be exposed to harmful chemicals as a result of industrial accidents, sabotage, or the intentional or unintentional actions of enemy or friendly forces." Therefore, an increasingly important issue in force protection will be toxicology exposure assessment associated with chemical and materiel exposures at uncharacterized deployed sites. Since deployed personnel may be exposed to multiple dynamic environmental changes at fixed sites, rapid assessment field-forward methodology for force readiness and health monitoring will be required. The development and application of identified biomarkers using the above-mentioned biotechnologies will help to identify the potential for serious injury to deployed warfighters exposed to toxic substances and environments by enabling identification of toxic effects within the warfighter before significant decrement can impact mission performance. Development of novel human markers of organ-selective exposure and monitoring methodologies that provide real-time detection of the potential for toxic injury and institution of protective countermeasures will minimize mission degradation due to environment-related adverse health effects (Fig. 1). This information can provide field commanders with fast and accurate toxicity information needed for force health protection and personal protective equipment selection and site selection decision making, thus minimizing individual deployment time in hazardous environments.

Current methods of determining toxic exposures to the warfighter are not adequate to prevent serious health effects or to predict and prevent low-level exposures that could have immediate performance or delayed health impacts. Simple chemical analysis of the environment is not sufficient to predict the effect of the myriad of combinations of diverse toxic substances at multiple exposure concentrations. The use of biomarker sets, or biosignatures, can be indicative of exposure to very low, subtoxic concentrations of hazardous substances before producing irreversible damage. One major challenge will be to identify Omic changes following toxic insults in this data-rich field of study that signify irreversible damage to human health compared to those changes that are temporary in nature and are resolved by the body's natural detoxification mechanisms without decreasing warfighter well-being and effectiveness. A second significant challenge will be to gain the commanders support for the collection of urine, saliva, sweat, blood, or epithelial cells for analysis in order to identify critical health events. A third major challenge will be identifying field-forward subject matter experts who can coordinate the collection, analysis, and interpretation of the biomarkers data to permit real-time decision making.

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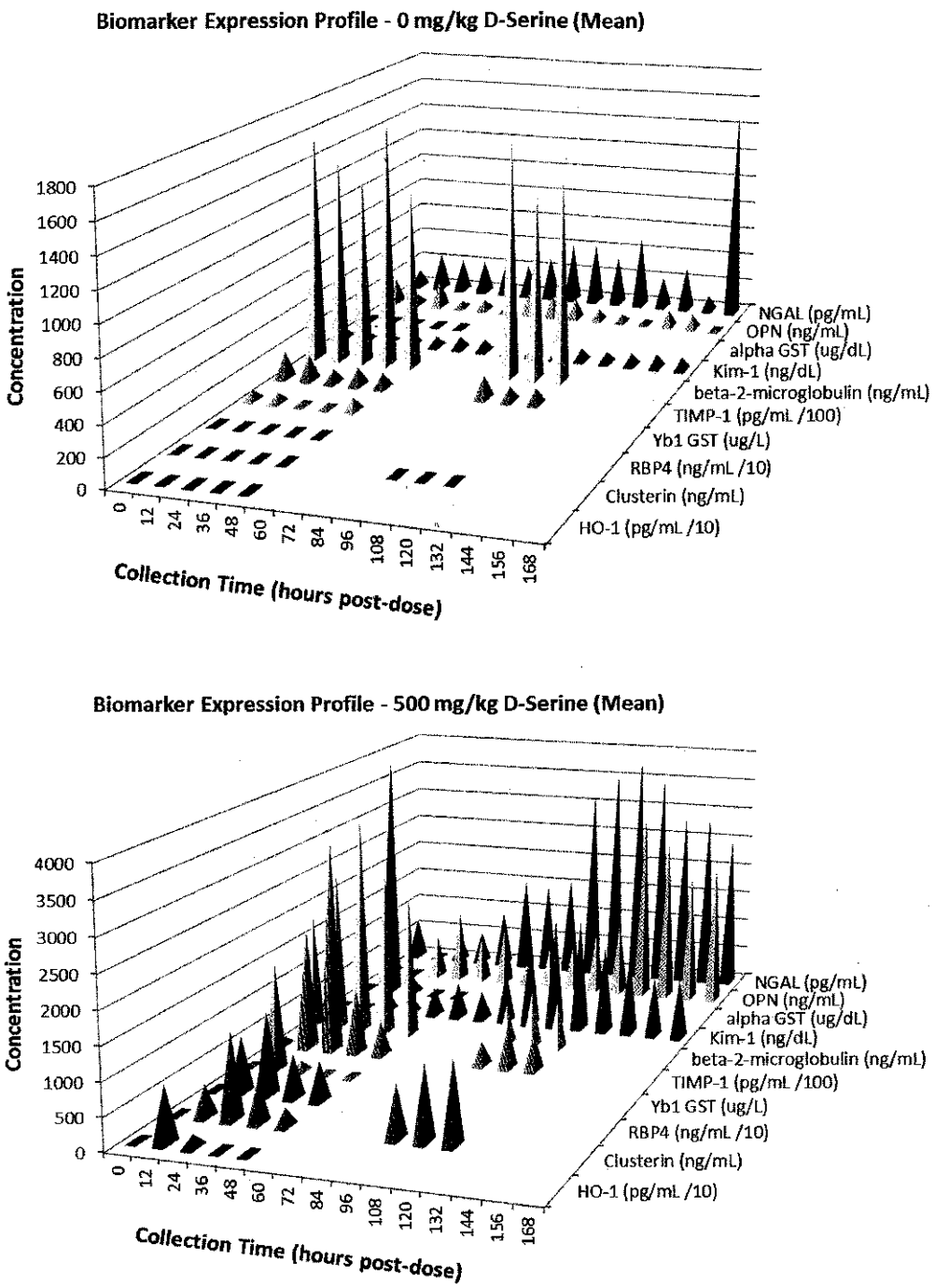

FIGURE 1. A representative signature set of urinary proteins collected over 168 hours following exposure to a known nephrotoxin. Control (top) and dosed (bottom). Proteins analyzed: HO-1 (heme oxygenase 1), RBP4 (retinol binding protein 4), Yb1 GST (Mu glutathione-*S*-transferase, rat equivalent of human γ GST), TIMP-1 (tissue inhibitor of metalloproteinases-1), Kim-1 (kidney injury molecule-1), α GST (α-glutathione-*S*-transferase), OPN (osteopontin), NGAL (neutrophil gelatinase–associated lipocalin). The nephrotoxin dose effect was anchored by histopathology data indicating low-level organ damage. Taken from Mauzy et al.[1]

It is widely believed that gene, protein, and metabolite expression changes are more accurate, reliable, sensitive, and informative as quantitative endpoints than the traditional clinical chemistry toxicity endpoints. Ultimately, such efforts may allow the identification of novel biomarkers for rapid monitoring and prediction of health hazards associated with chemical exposure. In addition, such work will help to identify gene, protein, and metabolic pathway targets for nutritional and pharmaceutical prophylactics as well as therapeutic intervention.

METABONOMICS AT AFRL

Metabonomics, a component of the broader field of metabolomics, is defined as "the quantitative measurement of the time-related multiparametric metabolic response of living

organisms to pathophysiological stimuli or genetic modification."[2] The field of metabonomics is concerned with the study of fixed cellular and biofluid concentrations of endogenous metabolites, as well as dynamic metabolite fluctuations, exogenous species, and molecules that arise from chemical rather than enzymatic processing.[3] Metabonomics is an approach used to characterize the metabolic profile of a specific tissue or biofluid, and is an attractive approach to the study of time-related metabolic responses to pathophysiological processes. A metabolic pathway alteration created by toxin exposure (or other stressor) is expressed as a "fingerprint" of biochemical perturbations[4,5] that is unique to the type of agent, altered structure, function, or resulting disease process (Fig. 2). These metabolic alterations are often seen in the urine as changes in metabolic profile in response to toxicity or disease as the body attempts to maintain homeostasis by eliminating substances from the body. Because metabolites are downstream of both gene transcription and enzyme activities, metabonomics has the potential to give a more accurate picture of the actual physiological state of an organism.

Both nuclear magnetic resonance (NMR) and liquid chromatography mass spectrophotometry (LC-MS)-based metabonomics are applied at AFRL and both have shown great promise as valuable tools for discovery of the metabolic response to tissue injury and chemical/physical stressors. NMR metabolic analysis using biofluids (i.e., blood, urine, saliva, etc.) is well documented, and the principles of this approach have been described in detail.[5–11] The use of LC-MS is complementary to data found using NMR, and is an additional tool that enhances metabonomics analysis for biomarker discovery.

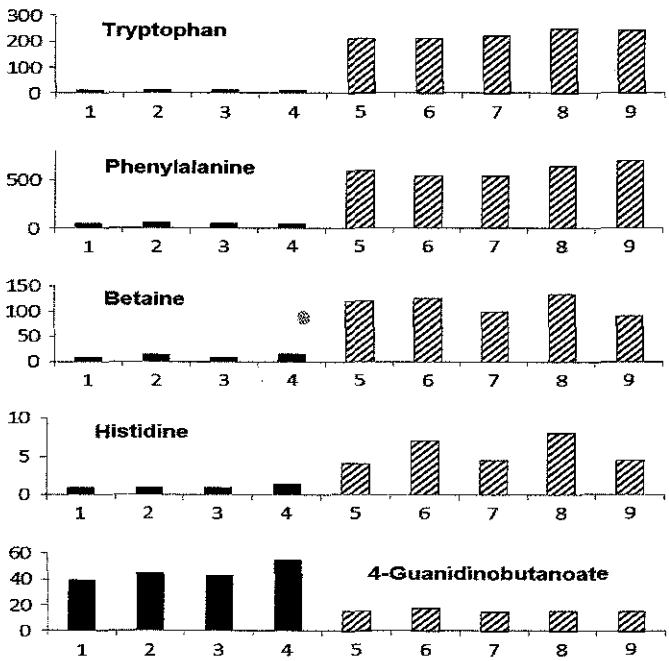

FIGURE 2. Metabolite biosignature of low-level kidney damage. Significantly altered metabolites were identified using LC-MS analysis of animals exposed to a known nephrotoxin (D-serine). Control animals (1–4; solid) and dosed animals (5–9; hashed) responses are shown. Taken from DelRaso et al.[42]

The advantages of NMR-based metabonomics include nondestructive analysis, applicable to intact biomaterial, and information rich with respect to determinations of molecular structure, especially in complex mixtures. The nonselectivity, lack of sample bias, and reproducibility of NMR[12] are of critical importance when considering toxicological screening applications. Changes in NMR-derived urinary metabolite levels have proven to be a sensitive indicator of chemical-induced toxicity.[10,13–15] LC-MS offers the ability to detect chemical classes not detected by NMR (i.e., sulfates), and the capability to detect lower abundance metabolites with little sample processing.[16] MS detection complements NMR analysis and enhances metabolite identification.

PROTEOMICS AT AFRL

The proteome in humans is orders of magnitude more complex than the genome. Numerous post-translational modifications combined with amino acid substitutions have been reported for the myriad of proteins coded by the genome. Discovering these differentially expressed protein profiles and their corresponding post-translational modifications are critical steps in understanding the molecular mechanisms regulating function in both single cells, organ system as well as in an organism. Quantitative proteomics detects minor changes in protein and/or peptide abundance in an organism in response to different kinds of altered states. Sample preparation is a critical first step in any protein profiling experiment, and both the source of the sample as well as the subsequent processing steps are greatly dependent on the targeted biomarker objectives of the study. Depletion of high-abundance proteins before LC-MS/MS, especially in blood samples, is essential to allow the ion signal of lower concentration analytes to be detected. It is within these low-abundance protein sets that markers are isolated, which have the highest likelihood of biological significance.

Two-dimensional (2D) gel electrophoresis has been used for over 30 years for protein separation[17] but a newer method, called 2D difference in-gel electrophoresis, can be used to minimize gel-to-gel variations by covalently labeling two samples and control with 3 different fluorescent dyes before gel separation. We have successfully used 2D difference in-gel electrophoresis approach to identify biomarkers of nephro and neurotoxicity.[18,19] Another emerging and powerful approach employs Beckman Coulter's Proteome PF 2D system (Beckman Coulter, Indianapolis, Indiana) combined with proprietary analogs.[20–24] PF 2D employs 2D separation using isoelectric focusing followed by reverse-phase high-pressure liquid chromatography. In addition, on-line electro spray ionization (ESI) time of flight mass spectrometry enables further detection in a third dimension.[25]

MS techniques used for protein identification can be classified by the type of mass spectrometer being utilized (quadrupole, time of flight, ion trap, ion cyclotron resonance etc. as well

as hybrid instruments). Different ionization techniques are usually used as a source and interface with the high-pressure liquid chromatography. ESI and matrix-assisted laser desorption ionization are the two major techniques commonly used by researchers for protein profiling. Nano spray offers a higher sensitivity than ESI[26,27] and has become the de facto standard for LC-MS-based proteomics. LC-MS-based experiments use two main approaches, labeled and label free.[28] Most of our proteomic analyses use relative and absolute quantification (iTRAQ, TMT), which use isobaric tags and permit comparison of up to 10 samples at the same time.[29–34] After the data are collected, they are analyzed via different suitable software packages for discovering protein biomarker from identified differentially detected peptides.

GENOMICS/EPIGENETICS AT AFRL

Toxicogenomics and epigenetics procedures are based on the use of high-throughput DNA microarray technology that determines the cellular response by examining RNA changes on exposure to chemicals. The AFRL Genomics Core Facility uses the Affymetrix GeneChip Technology, established in discovery work in 2001, and has provided technical support to many researchers in the Department of Defense (DoD). The initial toxicogenomics program was established to identify "toxicogenomic fingerprints" (i.e., the signatures of transcriptomic responses resulting from toxic chemical exposure) of the chemicals of interest to the Air Force. Since gene expression changes affect virtually all expressed genes, the genomic approach is significantly more sensitive than traditional biochemical assays or pathology techniques. Changes in RNA expression produce biomarkers that result from chemical exposure, especially at low doses and before the onset of pathological events. In addition, gene expression changes can provide mechanistic insight for a better understanding of the toxic response. This information also provides essential information needed to prevent exposure or mitigate toxicity related to environmental chemical exposures.

In our experience, chemical exposure effects that are detectable using traditional methods will generally result in expression changes of a few hundred genes. The investigation of the biological significance of such complex gene expression patterns involves a series of data mining and bioinformatics analyses. Initially, we use pattern recognition techniques (e.g., hierarchical clustering analysis) to group the coregulated genes, according to their temporal or dose response to the environmental chemical exposure.[35] Follow-on data analyses include use of bioinformatics tools with gene ontology and pathway analysis (Fig. 3), which organizes genes according to their biological function, permitting identification of biological consequences related to gene expression changes.

Chemical exposures generally result in perturbation of a large number of pathways. To provide a systems level understanding of chemical toxicity, we use bioinformatics tools to integrate the gene expression changes and pathway alterations into biological associated networks. Genomic data analysis can identify critical pathway perturbations, and bioinformatics analyses further reveals connection points of different pathways and how they are affected by the chemical exposure (Fig. 4). In addition, transcriptomic profiling allows identification of major regulators involved in the toxic response. These major regulators are normally presented as highly connected

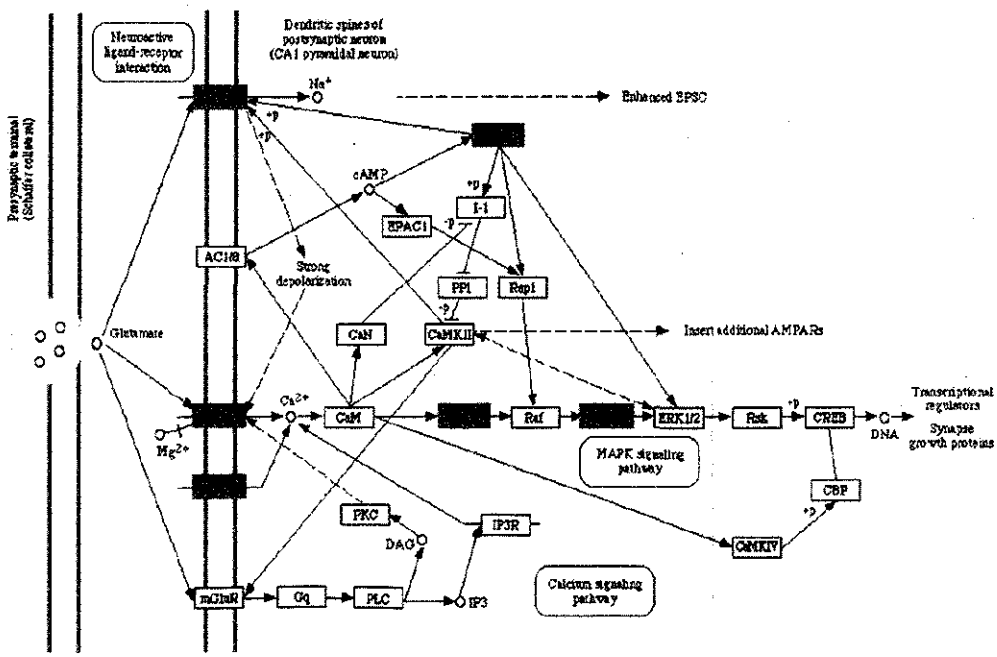

FIGURE 3. Example of pathway analysis of differentially expressed genes. The differentially expressed genes (blue boxes—all down regulated), resulted from neurotoxin (chlorpyrifos) exposure. Taken from Stapleton and Chan.[35]

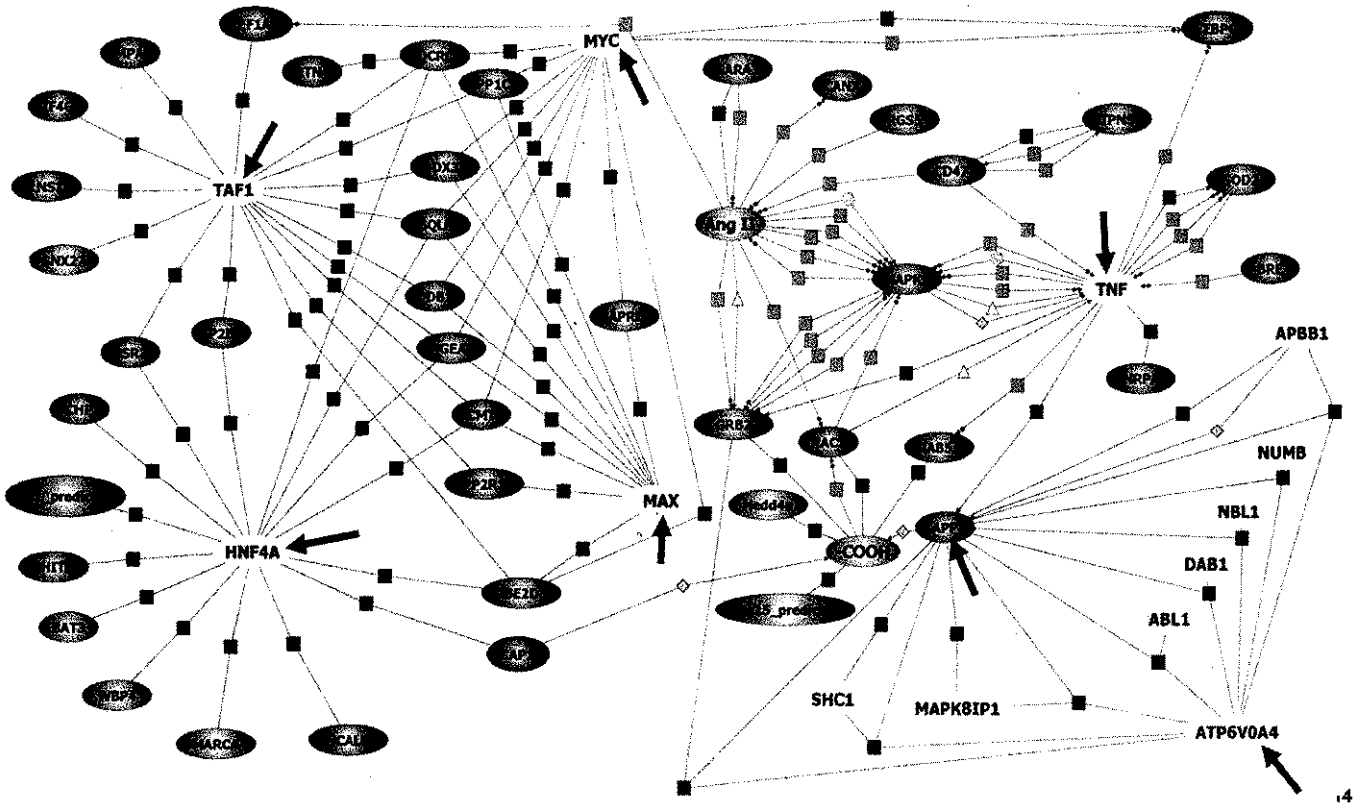

FIGURE 4. Example of a biological association network to differentially expressed genes. The connections between differentially expressed genes, genes with no expression changes, and small molecules are shown. The highly connected nodes are indicted by the green arrows. The network map is interactive that the scientific evidence supporting the connections can be accessed by clicking the small boxes on the edges. Red oval: up-regulated genes; blue oval: down-regulated genes; white oval: genes with no expression change; green oval: small molecules. Taken from Davidson et al.[37]

nodes in the network (i.e., the hubs of the network) (Fig. 4, green arrow). Modulating the levels of these regulators will thus have profound effects on the behavior of the network.

Using these methodologies, we have successfully used the transcriptomic profiling techniques to show that gene expression changes in whole blood can serve as novel biomarkers indicative of organ-specific damage resulting from hepatotoxin, neurotoxin, or nephrotoxin exposure.[36] These exposures resulted in unique patterns of gene expression detectable in whole blood. Further examination of these gene lists revealed that 40 genes were common in all 3 chemicals. Alterations in the expression profiles of these 40 genes can be used as a biomarker signature for exposed animals whose exposure type and level can be separated based on this pattern alone. This research demonstrated that whole blood is a suitable for monitoring changes in gene expression for the detection of organ-specific toxicity. To elucidate the molecular mechanisms of the toxicity of these chemicals, the gene expression changes in the target organs were used to construct pathway maps and biological association networks (Figs. 3 and 4). The findings of these analyses were subsequently published in peer-reviewed journals.[37–38]

We also used this approach to investigate the molecular mechanisms of nephrotoxicity. Kidney injury was induced by the use of four different nephrotoxins with regional specificity, and unique patterns of differential gene expression and pathway perturbation were identified.[39] Gene ontology and pathway analyses revealed perturbations of specific biological processes and pathways, which correlate with the phenotypic changes seen in clinical chemistry and histopathology analyses. These findings provided important insights into the molecular mechanisms of toxicity of these nephrotoxins.

In addition to the interrogation of the transcriptome, the AFRL Genomics Core Facility is fully capable of analyzing a variety of mechanisms involved in the regulation of the transcriptome, such as variances in genomic sequences (i.e., genetic polymorphisms), as well as DNA methylation, chromatin changes, and post-transcriptional regulations (including mRNA transport, processing, accessibility, and stability), collectively known as epigenetics.

AFRL OMICS APPLICATIONS

The use of Omics technologies has expanded from its initial use in toxicology exposure monitoring into several new areas of human performance and protection within AFRL. Below we describe three of the completed efforts within the biomarker discovery group. Not all projects use an Integrated Omics approach which, although encompassing and data rich, can be expensive to execute. Biomarkers, once identified

through Omics analyses, require prevalidation and validation within animal and human cohort samples.

Exposures to Toxic Substances (DTO)

To accomplish the DTO previously described, a combination of toxicogenomics, proteomics, and metabonomics analyses of exposure study samples were used in the biomarker discovery process to identify markers to organ-selective toxic effects (i.e., alterations in gene, protein, and metabolite expression) to specific chemical exposure(s). While not initiated in this project, validation of biomarker/biomarker panels usually follows the discovery phase and involves standardization of assay procedures for analytic validity as well as full human cohort testing.[39–41] A biomarker signature, once validated, could be used as a biomolecular screening tool to identify adverse effects of hazardous exposure to military personnel before manifestation of significant decrement that could impact mission performance. This project examined marker patterns in urine/blood samples from animals exposed to a number of well-characterized nephrotoxins and hepatotoxins at varying doses. As a result, a set of potential unique target organ biomarkers/biomolecular signatures of chemical exposure were identified using urine and blood samples.[18,19,42–45] These new markers, when used in conjunction with the prevalidation of literature-based markers (Fig. 1), could provide a fast and minimally to noninvasive method to use the host response to quickly monitor exposure status. As seen with our preliminary data, it may be possible to use the biosignatures not only to evaluate status in response to exposure, but also to use the response time of a subset of these markers to determine "when" the exposure took place. In theory, if linked to Global Positioning System data identifying the individual's location these data could also indicate "location" of exposure.

Jet Fuel/Exhaust Exposure

Due to its wide spread use, JP-8 has been recognized as the single largest source of chemical exposure for U.S. and NATO military personnel[46]; inhalation and dermal have been shown to represent the primary routes of exposure.[47] This has resulted in the potential for widespread occupational exposure among military and civilian personnel that may result in toxicity to the immune system, nervous system, and respiratory tract.[48] Therefore, a tremendous need exists to identify biomarkers predictive of toxic insult due to exposure of volatile organic chemicals. Metabonomics analysis offers a minimally invasive sample collection, minimal sample processing, robust and stable analytical platform, with excellent analytical and biological reproducibility that has the potential to identify biomarkers predictive of hazardous flight line exposures.

We compared urine samples obtained from Japanese and U.S. flight line personnel stationed at a number of air bases in Japan using NMR-based metabonomics analysis to identify potential biomarkers of exposure to JP-4 and JP-8 fuels and/or their combustion products. Urine samples were obtained from air bases servicing F-15 and C-130 aircraft that were fueled with JP-4 (Japanese) and JP-8 (United States). Previous studies have indicated that naphthalene, 1-naphthol, and 2-naphthol would be good potential biomarkers of exposure to JP-8.[49–51] However, these studies were conducted using subjects who worked in fuel tanks where exposures to JP-8 volatile organic carbons would be high. No potential biomarkers have been reported that would indicate exposure to JP-4. Since the U.S. military has transitioned from JP-4 to JP-8 use in their aircraft since 1969, it is unlikely that much research has been conducted to monitor for exposure to JP-4.

Our NMR-based metabonomics approach to study flight line personnel exposed to jet fuel or to its combustion products, demonstrated observable differences in urinary metabolite profiles that could be distinguished from unexposed control subjects.[52] Furthermore, incorporation of metadata into the metabonomics data analysis was found to increase the discriminatory power approximately 2-fold as well as predictive accuracy. An important contributing factor appeared to be related to the type of aircraft. Personnel working with F-15 aircraft showed greater effects on urinary metabolite profiles than those working with C-130 aircraft, regardless of fuel type (JP-4 or JP-8). Discriminant analysis of multivariate data was able to classify flight line personnel from control subjects with statistical significance ($p < 0.01$). Studies are continuing to identify as many urinary metabolites as possible that lead to group exposure classification, and may provide a set of biomarkers predictive of jet fuel/exhaust exposure.

Cognitive Fatigue

Urine is a noninvasively collected, information-rich biofluid that can provide insight into the metabolic state of an organism and as a result, is often a focus in metabonomics investigations. Targeted profiling is a powerful tool that can drive such studies, providing direct identification and quantification of a variety of potential metabolite biomarkers. This approach was demonstrated in the previous DTO work described above, as well as in previous animal toxicity studies by other researchers. Statistically significant group classification based on NMR spectral analysis has been previously shown by Clayton et al[53] in a metabonomics study investigating the susceptibility of rats to acetaminophen toxicity. This work described an alternative and conceptually new "pharmaco-metabonomic" approach to personalizing drug treatment that used a combination of pretreatment metabolite profiling and chemometrics to model and predict the responses of individual subjects. This study was able to predict sensitivity/resistance to acetaminophen toxicity in rats before exposure to the compound.

Using Clayton's approach[53] applied to human fatigue susceptibility, our preliminary results indicated that urinary metabolite profiles identified using NMR and LC-MS analyses were able to classify subjects with respect to cognitive performance decrement during sleep deprivation–induced fatigue. Results obtained from the cognitive fatigue study also indicated that

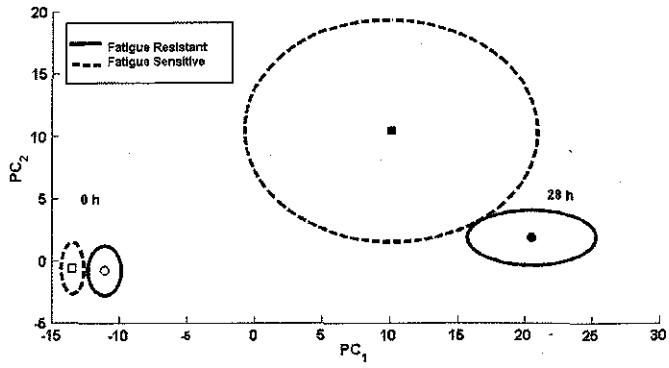

FIGURE 5. Principal Component Analysis scores plot separation of urinary metabolites from fatigue-resistant and fatigue-sensitive human subjects as a function of sleep deprivation time. Fatigue-resistant (circles) and fatigue-sensitive (squares) data are plotted at 0 hour (open symbols) and 28 hours (filled symbols) of sleep deprivation. Such data indicate that metabolite signatures can be used to predict future status. Taken from DelRaso et al.[54]

baseline NMR spectral profiling of human urine and chemometric analysis predicted cognitive fatigue susceptibility (Fig. 5). A subsequent Air Force sleep deprivation study was conducted to determine if differences in individual susceptibility to sleep deprivation–induced fatigue could be discerned, and if biomarkers associated with fatigue resistance/susceptibility could be identified.

Recent advances in our laboratory's bioinformatics tools development for multivariate statistical analysis of NMR-based metabonomics data has resulted in more accurate biosignature and biomarker identification. In a recent sleep deprivation study, bioinformatic analysis of NMR data indicated that urinary metabolite profiles could discern differences in cognitive performance of study subjects 12 hour before the cognitive testing phase, and of subjects that were sleep-deprived following 28 hours of continuous wakefulness.[54] Furthermore, psychological cognitive testing scores (i.e., psychomotor vigilance test [PVT] and Automated Neuropsychological Assessment Metricscore), with regard to classification as cognitively resistant or sensitive to sleep deprivation–induced fatigue, were found to correlate with urinary metabolite profiles determined at 28 hours of sleep deprivation. The PVT is the standard psychological testing method for assessing cognitive fatigue and it is well established that peak fatigue occurs following 28 h of sleep deprivation. The results from the NMR-based metabonomics analysis of human urine suggest that urinary metabolite profiling may also be useful as a classifier of cognitive function under conditions of sleep deprivation–induced fatigue. Currently, none of the fatigue detection technologies developed (e.g., electroencephalography, pupillometry, multiple sleep latency test, PVT, and Automated Neuropsychological Assessment Metrics-core) has been adequately verified in an operational environment, nor has it been shown that these technologies can predict the impact of fatigue on future cognitive performance.

The results of our study demonstrated that NMR-based metabonomics could be used in a noninvasive way to predict and characterize sleep deprivation–induced fatigue impact on cognitive performance. Furthermore, our results indicated that NMR-based metabonomics analysis of human urine identified changes in urinary metabolite profiles that corresponded to psychological testing scores of cognitive performance decrement under sleep deprivation–induced fatigue. More importantly, NMR-based metabonomics was also capable of predicting susceptibility to sleep deprivation–induced fatigue 12 hours before actual testing in the 24 hour cognitive testing phase of the study; PVT analysis failed to detect any significant differences in cognitive performance at this time point.

FUTURE OF INTEGRATED OMICS FOR DOD TOXICOLOGY APPLICATIONS

As with most technologies, Omics technologies will not be the "be-all and end-all" methods for addressing all issues related to toxicity and health of our service men and women. However, it certainly will add value in many areas of biological research. Metabonomics has shown particular promise in the area of toxicology. It is anticipated that metabonomics will play a crucial role in the integration of the other Omics sciences (i.e., genomics and proteomics). Because metabolites reflect actual biochemical events that were initiated at the genome level, they can be used in conjunction with proteomic and genomic data to "walk backwards" to identify what proteins were translated and from what genes they were transcribed.

Clearly, Integrated Omics can be used in the future to address health issues using various biofluid and tissue samples collected and stored in military biological repositories. Our research, described above, has demonstrated the significant potential of Omics to advance mechanistic toxicological and psychological research in the DoD. We are getting closer, but further research is needed to realize the full potential of Omics applications in force health protection through early detection of potential adverse effects in military service members who are exposed to hazardous materials or stressors before they cause a reduction in health and operational performance decrement.

REFERENCES

1. Mauzy CA, Todd D, Rono V, Frey J, Fleming E, Kaziska D: Biomarkers of Exposure to Toxic Substances. Vol. 5: Biomarker Prevalidation Studies. Prevalidation of Urine and Serum Biomarkers Indicative of Subclinical Kidney Damage in a Nephrotoxin Model. Air Force Research Laboratory Technical Report AFRL-RH-WP-TR-2009-0106. Dayton, OH, Wright-Patterson AFB, 2010. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a535835.pdf; accessed May 22, 2014.
2. Nicholson JK, Lindon JC, Holmes E: 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999; 29: 1181–9.
3. Lindon JC, Holmes E, Nicholson JK: So what's the deal with metabonomics? Anal Chem 2003; 75: 385A–91A.
4. La S, Yoo HH, Kim, D-H: Liquid chromatography-mass spectrometric analysis of urinary metabolites and their pattern recognition for the prediction of drug-induced hepatotoxicity. Chem Res Toxicol 2005; 18: 1887–96.
5. Robertson DG: Metabonomics in toxicology: a review. Toxicol Sci 2005; 85: 809–22.

6. Lindon JC, Holmes E, Nicholson JK: Toxicological applications of magnetic resonance. Prog Nucl Mag Reson Spec 2004; 45: 109–43.
7. Lenz E, Bright J, Wilson I, Morgan S, Nash A: A 1H NMR-based metabonomic study of urine and plasma samples obtained from healthy human subjects. J Pharm Biomed Anal 2003; 33: 1103–15.
8. Reo NV: NMR-based Metabolomics. Drug Chem Toxicol 2002; 25: 375–82.
9. Holmes E, Nicholls AW, Lindon JC, et al: Chemometric models for toxicity clarification based on NMR spectra of biofluids. Chem Res Toxicol 2000; 13: 471–8.
10. Holmes E, Shockcor JP: Accelerated toxicity screening using NMR and pattern recognition-based methods. Curr Opin Drug Discovery Dev 2000; 3: 72–8.
11. Holmes E, Nicholl AW, Lindon JC, et al: Development of a model for classification of toxin-induced lesions using 1H NMR spectroscopy of urine combined with pattern recognition. NMR Biomed 1998; 11: 235–44.
12. Keun HC, Ebbels TM, Antti H, et al: Analytical reproducibility in (1)H NMR-based metabonomic urinalysis. Chem Res Toxicol 2000; 15: 1380–86.
13. Robertson DG, Reily MD, Sigler RE, Wells DF, Paterson DA, Braden TK: Metabonomics: evaluation of nuclear magnetic resonance (NMR) and pattern recognition technology for rapid in vivo screening of liver and kidney toxicants. Toxicol Sci 2000; 57: 326–37.
14. Waters NJ, Holmes E, Waterfield CJ, Farrant RD, Nicholson JK: NMR and pattern recognition studies on liver extracts and intact livers from rats treated with alpha-naphthylisothiocyanate. Biochem Pharmacol 2002; 64: 67–77.
15. Nicholson JK, Connelly J, Lindon JC, Holmes E: Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 2002; 1: 153–61.
16. Dunn WB, Ellis DI: Metabolomics: current analytical platforms and methodologies. Trend Anal Chem 2005; 24: 285–94.
17. O'Farrell P: High resolution two dimensional electrophoresis of proteins. J Biol Chem 1975; 250: 4007–21.
18. Shiyanov P, Pitsch R, Qi L, Chen J: Biomarkers of Exposure to Toxic Substances, Vol. 3: Proteomics; Biomarkers to Kidney and Organ Damage. Air Force Research Laboratory Technical Report AFRL-RH-WP-TR-2009-0104. Dayton, OH, Wright-Patterson AFB, 2009. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a521156.pdf; accessed May 22, 2014.
19. Woolard CL, Mauzy CA, Shiyanov PA: Biomarkers of Exposure to Toxic Substances, Vol. 7: Identification of Potential Serum Protein Biomarkers Indicative of Low Level Kidney Degradation in Response to Toxin Exposures. Air Force Research Laboratory Technical Report AFRL-RH-WP-TR-2010-0146. Dayton, OH, Wright-Patterson AFB, 2010. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a535840.pdf; accessed May 22, 2014.
20. Simonian MH, Betgovargez E: Proteome analysis of human plasma with the ProteomeLab PF 2D system. Beckman Coulter Application Information Bulletin A-1963A. 2003. Located at Jaz D Lifesciences. Available at http://www.jazdlifesciences.com/pharmatech/research/Beckman-Coulter-Inc.htm?contentSetId=34692; accessed February 18, 2015.
21. Billecke C, Malik I, Movsisyan SS, et al: Analysis of glioma cell platinum response by metacomparison of two-dimensional chromatographic proteome profiles. Mol Cell Proteomics 2006; 5: 35–42.
22. Linke T, Ross AC, Harrison EH: Proteomic analysis of rat plasma by two-dimensional liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. J Chromatogr A 2006; 1123(2): 160–9.
23. Lubman DM, Kachman MT, Wang H, et al: Two-dimensional separations—mass mapping of proteins from human cancer cell lysates. J Chromatogr B 2002; 782: 183–96.
24. Wang Y, Wu R, Cho KR, Shedden KA, Barder TJ, Lubman DM: Classification of cancer cell lines using an automated two-dimensional liquid mapping method with hierarchical clustering techniques. Mol Cell Proteomics 2006; 5: 43–52.
25. Wall DB, Parus SF, Lubman DM: Three-dimensional protein map according to pI, hydrophobicity and molecular mass. J Chromatogr B 2002; 774(1): 53–8.
26. Wilm M, Mann M: Analytical properties of the nanoelectrospray ion source. Anal Chem 1996; 68: 1–8.
27. Wilm M, Shevchenko A, Houthaeve T, et al: Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 1996; 379: 466–69.
28. Ong SE, Blagoev B, Kratchmarova I, et al: Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 2002; 1(5): 376–86.
29. Thompson A, Schäfer J, Kuhn K, et al: Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 2003; 75(8): 1895–904.
30. Ross PL, Huang YN, Marchese JN, et al: Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004; 3(12): 1154–69.
31. Wiese S, Reidegeld KA, Meyer HE, Warscheid B: Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. Proteomics 2007; 7(3): 340–50.
32. Aggarwal K, Choe LH, Lee KH: Shotgun proteomics using the iTRAQ isobaric tags. Brief Funct Genomic Proteomic 2006; 5(2): 112–20.
33. Murphy JP, Everley RA, Coloff JL, Gygi SP: Combining amine metabolomics and quantitative proteomics of cancer cells using derivatization with isobaric tags. Anal Chem 2014; 86(7): 3585–93.
34. Elliott MH, Smith DS, Parker CE, Borchers C: Current trends in quantitative proteomics. J Mass Spec 2009; 44(12): 1637–60.
35. Stapleton AR, Chan VT: Subtoxic chlorpyrifos treatment resulted in differential expression of genes implicated in neurological functions and development. Arch Toxicol 2009; 83(4): 319–33.
36. Chan V, Stapleton A, Soto A, Yu K, DelRaso N: Identification of Gene Expression Changes in Whole Blood Indicative of Exposure to Chemicals with Different Target Organ Toxicity. Air Force Technical Report AFRL-HE-WP-TP-2006-0032. Dayton, OH, Wright-Patterson AFB, 2006. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a457815.pdf; accessed May 22, 2014.
37. Davidson ME, Kerepesi LA, Soto A, Chan VT: D-Serine exposure resulted in gene expression changes implicated in neurodegenerative disorders and neuronal dysfunction in male Fischer 344 rats. Arch Toxicol 2009; 83(8): 747–62.
38. Soto A, DelRaso NJ, Schlager JJ, Chan VT: D-Serine exposure resulted in gene expression changes indicative of activation of fibrogenic pathways and down-regulation of energy metabolism and oxidative stress response. Toxicology 2008; 243(1–2): 177–92.
39. Hunter DJ, Losina E, Guermazi A, Burstein D, Lassere MN, Kraus V: A pathway and approach to biomarker validation and qualification for osteoarthritis clinical trials. Curr Drug Targets 2010; 11(5): 536–45.
40. Katja Köhler K, Seitz H: Validation processes of protein biomarkers in serum—a cross platform comparison. Sensors 2012; 12: 12710–28.
41. Atashpaz-Gargari E, Braga-Neto UM, Dougherty ER: Modeling and systematic analysis of biomarker validation using selected reaction monitoring. EURASIP J Bioinform Syst Biol 2014; 2014: 17.
42. Chan V, Soto A, Yu K, Stapleton A, Davidson M: Biomarkers of Exposure to Toxic Substances, Vol. 2: Genomics; Unique Patterns of Differential Gene Expression and Pathway Perturbation Resulting from Exposure to Nephrotoxins with Regional Specific Toxicity. Air Force Technical Report AFRL-RH-WP-TR-2009-0103. Dayton, OH, Wright-Patterson AFB, 2009. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a531077.pdf; accessed June 19, 2014.
43. DelRaso N, Mahle D, Rietcheck R, et al: Biomarkers of Exposure to Toxic Substances, Vol. 6: Dose and Time Response of Liver Toxicant. Air Force Technical Report AFRL-RH-WP-TR-2010-0143. Dayton, OH, Wright-Patterson AFB, 2010. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a534170.pdf; accessed June 19, 2014.
44. DelRaso N, Mauzy C, Rietcheck R: Biomarkers of Exposure to Toxic Substances, Vol. 1: Global Experimental Design: Biomarker Discovery

for Early Prediction of Organ-Selective Toxicity. Air Force Technical Report AFRL-RH-WP-TR-2009-0102.Dayton, OH, Wright-Patterson AFB, 2009. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a606414.pdf; accessed December 30, 2014.

45. DelRaso N, Reitcheck R, Mahle D, et al: Biomarkers of Exposure to Toxic Substances, Vol. 4: Metabonomics Biomarkers to Liver and Organ Damage. Air Force Research Laboratory Technical Report AFRL-RH-WP-TR-2009-0105. Dayton, OH, Wright-Patterson AFB, 2009. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a530977.pdf; accessed June 19, 2014.
46. Carlton GN, Smith LB. Exposures to jet fuel and benzene during aircraft fuel tank repair in the US Air Force. Appl Occup Environ Hyg 2000; 15(6): 485–91.
47. Chao YC, Gibson RL, Nylander-French LA. Dermal exposure to jet fuel (JP-8) in US Air Force personnel. Ann Occup Hyg 2005; 49(7): 639–45.
48. National Research Council. Toxicologic assessment of jet-propulsion fuel 8. National Research Council, National Academy of Sciences, Washington, DC, 2003. Available at http://www.ncbi.nlm.nih.gov/books/NBK207629/; accessed February 18, 2015.
49. Serdar B, Egeghy PP, Waidyanatha S, Gibson R, Rappaport SM: Urinary biomarkers of exposure to jet fuel (JP-8). Environ Health Perspect 2003; 111(14): 1760–64.
50. Serdar B, Egeghy PP, Gibson R, Rappaport SM: Dose-dependent production of urinary naphthols among workers exposed to jet fuel (JP-8). Am J Ind Med 2004; 46(3): 234–44.
51. Smith KW, Proctor SP, Ozonoff A, McClean MD: Urinary biomarkers of occupational jet fuel exposure among air force personnel. J Exposure Sci Environ Epidemiol 2012; 22: 35–45.
52. DelRaso N, Mattie D, Kobayashi A, et al: Metabolomics characterization of U.S. and Japanese F-15 and C-130 flight line crews exposed to jet fuel volatile organic compounds and aerosols. AFRL-RH-WP-TR-2014-0140.
53. Clayton TA, Lindon JC, Cloarec O, et al: Pharmaco-metabonomic phenotyping and personalized drug treatment. Nature 2006; 440: 1073–77.
54. DelRaso N, Mahle D, Schlager J, et al: Biomarkers of Fatigue: Metabolomic Profiles Predictive of Cognitive Performance. Air Force Technical Report AFRL-RH-WP-TR-2013-009. Dayton, OH, Wright-Patterson AFB, 2013. Available at http://www.dtic.mil/dtic/tr/fulltext/u2/a594580.pdf; accessed June 4, 2014.